Thesis for doctoral degree (Ph.D.)
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based on biomarkers, phenotypes and new surgical thinking.

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A PERSONALIZED APPROACH TO CHRONIC RHINOSINUSITIS WITH NASAL POLYPS

BASED ON BIOMARKERS, PHENOTYPES AND NEW SURGICAL THINKING

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Till farmor

ABSTRACT

Chronic rhinosinusitis (CRS) is a prevalent disease causing a substantial burden for the patient and the society. CRS is divided into CRS with (CRSwNP) and without nasal polyps (CRSsNP). Based on current knowledge on inflammatory markers, CRS can be further divided into endotypes, CRSsNP mainly being characterized by a neutrophilic type 1 inflammatory response and CRSwNP being characterized by an eosinophilic type 2 inflammation. With the increase of type 2 inflammation in CRSwNP patients, asthma comorbidity and relapse of disease becomes more frequent. In recent years, monoclonal antibodies (mAbs) directed towards the type 2 inflammatory response have been demonstrated to be efficacious in CRSwNP.

The overall goal of this thesis was to investigate the pathophysiology of CRS and to evaluate effects of novel treatments.

Paper I and II focus on biomarkers and clinical characteristics to help identify type 2 CRSwNP. Serum periostin can, together with serum IgE and *Staphylococcus aureus* enterotoxin (SE)-IgE, identify formation of IL-5 and SE-IgE in nasal polyp tissue with a reasonable sensitivity and specificity. Eosinophilic blood count correlates poorly with inflammatory markers in nasal polyp tissue, but can, together with clinical history of asthma, allergy and/or aspirin exacerbated respiratory disease, help identify most type 2 CRSwNP patients in a clinical setting. Furthermore, in paper II we show that a shift towards an increase in type 2 inflammation is seen in CRS over recent years in Central Europe, measurable both as an increase in inflammatory markers and as a shift of endotype. This shift is seen in non-asthmatic, non-allergic patients and is more pronounced in patients with CRSwNP than in patients with CRSsNP. The results also indicate that polyp formation, at least in part, is driven by mechanisms not directly related to the type and extent of tissue inflammation.

Paper III-V focus on novel treatment strategies. Paper III shows that treatment with dupilumab, a mAb directed to the IL-4Receptor α, reduces local type 2 inflammatory parameters in nasal secretions and nasal polyp tissue. Paper IV-V focus on reboot surgery. Reboot surgery appears to be favorable in terms of relapse in moderate to severe CRSwNP compared to conventional surgery, and it reduces type 2 inflammatory markers in nasal secretions, 12 months after surgery, in the same magnitude as dupilumab. The inflammation in CRSwNP appears to be widespread, involving not only polyps but also the seemingly healthy mucosa that lines the sinuses, a finding that strengthen the rationale for reboot surgery.

In summary, the described shift towards type 2 inflammation in CRS suggests that the disease is undergoing a continuous endotypic change towards a more severe state of disease. It is evident that carefully chosen biomarkers in combination with clinical characteristics can be used to identify type 2 CRSwNP. Reboot surgery is favorable compared to conventional surgery, and may, based on endotypes, as mAbs, be implemented in treatment for CRSwNP.

LIST OF SCIENTIFIC PAPERS

I Jonstam K, Westman M, Holtappels G, Holweg CTJ, Bachert C. **Serum periostin, IgE, and SE-IgE can be used as biomarkers to identify moderate to severe chronic rhinosinusitis with nasal polyps.** J Allergy Clin Immunol 2017; 140:1705-8.e3.

II Jonstam K, Delemarre T, Holtappels G, Cardell L-O, Westman M, Bachert C. **Type 2 inflammatory shift in nasal polyposis.** – Manuscript

III Jonstam K, Swanson BN, Mannent LP, Cardell LO, Tian N, Wang Y, et al. **Dupilumab reduces local type 2 pro-inflammatory biomarkers in chronic rhinosinusitis with nasal polyposis.** Allergy 2019; 74:743-52.

IV Alsharif S, Jonstam K, van Zele T, Gevaert P, Holtappels G, Bachert C. **Endoscopic Sinus Surgery for Type-2 CRS wNP: An Endotype-Based Retrospective Study.** Laryngoscope 2019.

V Jonstam K, Alsharif S, Bogaert S, Suchonos N, Holtappels G, Jae-Hyun Park J, Bachert C. **Extensive type 2 inflammation in chronic rhinosinusitis with nasal polyps is suppressed by complete sinus mucosa removal (reboot surgery).** – Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AERD	Aspirin exacerbated respiratory disease
AUC	Area under the concentration versus time curve
CT	Computed tomography
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
ECP	Eosinophil cationic protein
ELISA	Enzyme-linked immunosorbent assay
FESS	Functional endoscopic sinus surgery
GA^2LEN	Global asthma and allergy european network
GCS	Glucocorticosteroid
IgE	Immunoglobulin E
IL	Interleukin
IL-4Rα	Interleukin-4 receptor alpha
IL-13Rα	Interleukin-13 receptor alpha
IQR	Intraquartile range
IU	International units
LS	Least squares
mAb	Monoclonal antibody
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
PARC	Pulmonary and activated regulatory cytokine
QoL	Quality of life
RAND-36	Health related quality of life questionaire
SE	Standard error
SE-IgE	IgE antibodies to *S. aureus* enterotoxins
SNOT-22	22-item sino-nasal outcome test
Th	T helper cell

1 AIMS

The overall aim of this thesis is to investigate the pathophysiology of CRS and to evaluate the effects of novel treatments.

More specific to:

- Characterize the mucosal inflammation in CRSwNP (paper II and IV).
- Determine if the balance between type 1 and type 2 inflammation in CRS has changed in Central Europe during the past 8-10 years (paper II).
- Identify new biomarkers in serum and comorbidities useable for further endotyping CRSwNP (paper I and II).
- Assess the local treatment effect of a novel monoclonal antibody (dupilumab) in treatment of CRSwNP by analysing inflammatory markers in nasal secretion and polyp tissue (paper III)
- Appraise the clinical effect of a new surgical technique (reboot) in conjunction with evaluation its effect on local inflammation (paper IV and V)

2 INTRODUCTION

2.1 Chronic rhinosinusitis

Chronic rhinosinusitis (CRS) is a disease involving the nose and paranasal sinuses. The human has 4 sets of paired sinuses, the maxillary, the frontal, the ethmoid and the sphenoid sinuses. CRS, according to the European Positions Paper on Rhinosinusitis and nasal polyps (EPOS), is defined as any inflammation in the nose or the paranasal sinuses with symptoms prevalent for more than 12 subsequent weeks. The patient shall suffer from at least two symptoms, one being either nasal blockage/obstruction/congestion or nasal discharge, other symptoms can be facial pain/pressure and reduction or loss of smell. The symptoms shall be supported by either endoscopic signs of nasal polyps or mucopurulent discharge/oedema in the middle meatus and/or CT changes within the ostiomeatal complex or sinuses[1]. CRS is a common disease, the Global Asthma and Allergy European Network (GA^2LEN) study reported a prevalence of 10.9% in the European population [2], a study in the US showed that 11.9% of the population met the criteria for CRS with a peak in prevalence (15.9%) in the age group 50-59 [3]. CRS causes a substantial economic burden, both on direct[4] and indirect costs[5], the over-all direct cost of CRS is estimated to $10–$13 billion per year[6] in the US. CRS patients report a considerable impact on quality of life (QoL), both on disease specific tests[7, 8] and on generic QoL tests. These patients experience a noticeable impact on extra-rhinologic symptoms such as difficulty to sleep, cognitive dysfunction and overall productivity [9] and studies have shown that QoL scores of patients with CRS are in the same range or below other chronic diseases such as congestive heart failure, coronary artery disease and Parkinson's disease [10].

2.2 Pathophysiology

During many years, the term CRS has been used for any kind of chronic inflammation in the nose and sinuses, implying one homogeneous disease. Recent findings[11, 12] have pointed to a more complex picture, with different phenotypes and endotypes within the CRS group, with completely different histological and inflammatory patterns. Phenotypes are defined as measurable or observable traits or characteristics; in CRS, two different phenotypes are quite easy to identify with an endoscope, CRS with Nasal Polyps (CRSwNP) and CRS without Nasal Polyps (CRSsNP). In contrast, the definition of CRS endotypes are based on specific pathophysiological mechanisms resulting in different inflammatory responses [12, 13].

2.3 The defence system

The skin and the mucosa constitute the first line of defence, providing a physical barrier. The nose and the sinuses are covered by a mucosa consisting of three layers; the mucus, the epithelium with columnar ciliated respiratory epithelial cells, goblet

cells and basal cells, and the basement membrane[14]. The epithelium clears pathogens and particles from inhaled air by mucociliary clerance[15] and produces substances that have anti-pathogenic effects[16]. When chronically exposed to pathogens, the epithelium secrets chemokines and cytokines to activate inflammatory pathways and recruit immune cells[17, 18] (figure 1). The main focus of the immune system is to protect the body against harmful substances and pathogens. It can be divided in two different parts, the innate and the adaptive immune systems. The innate immune system consists of the cellular system which includes the actions of dendritic cells, monocytes, macrophages and granulocytes (eosinophils, neutrophils and basophils). The cellular response is activated when the pathogen evades the barrier. The innate immune system mediates a rapid protection against pathogens. It is activated upon stimulation with environmental factors including infectious agents and its response is rapid, within hours, and does not need to be specifically instructed. The adaptive immune system is slow to react but is more specific and long-lasting. It consists of two different types of lymphocytes, B and T cells. Naïve B and T cells circulate in blood and lymphoid tissue until they are activated by antigen presenting cells such as dendritic cells. Each naïve lymphocyte carries antigen receptors unique to one antigen. B-cells, when activated, differentiate into antibody secreting plasma cells or memory B-cells. T-cells are either cytotoxic T-cells or T helper cells (Th) secreting different cytokines depending on the type of Th cell (Th1, Th2, Th17)[19]. The immune system contains many control mechanisms and a disruption of these systems causes uncontrolled excess inflammation, as in CRS.

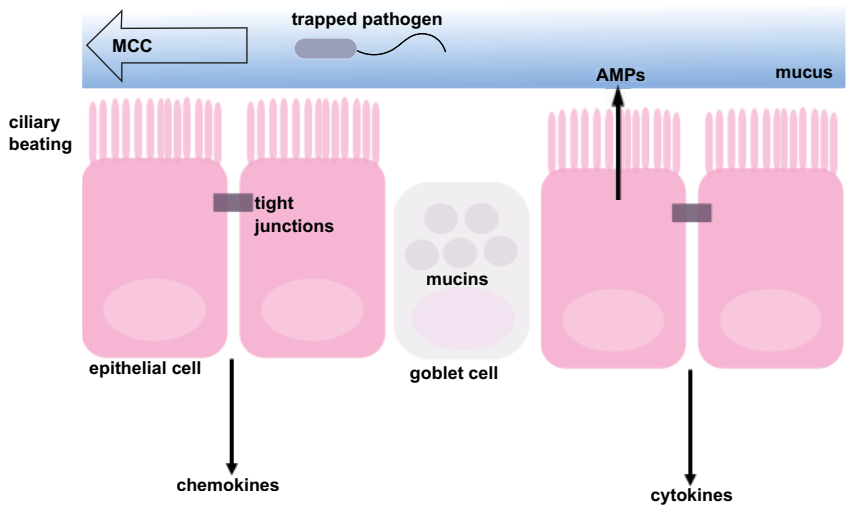


Figure 1: A schematic overview of the mucosal barrier in the sino-nasal tract. MCC: Mucociliary clearance AMPs; Anti-Microbial Peptides.

2.4 Mucosal inflammation

The underlying inflammation differs between phenotypes, i.e. CRSwNP and CRSsNP, and between different regions in the world. In western Europe, CRSsNP is mainly a type 1 neutrophilic inflammation with elevated levels of type 1 derived cytokines such as Interferon-γ, Interleukin (IL)-1β, IL-6, IL-8 and myeloperoxidase (MPO)[20]. On the other hand, CRSwNP in western countries, is mainly characterised by a type 2 inflammation, dominated by cytokines that induce an eosinophilic inflammation, characterized by the production of Immunoglobulin E (IgE), IL-4, IL-5, IL-13, Eosinophil Cationic Protein (ECP), eotaxin-2 and eotaxin-3, locally and systemically [21, 22]. Based on inflammatory markers, 10 different clusters of CRS have been identified, they can be divided into 3 different endotypes, types 1 to 3. Type 1, clinically resembling CRSsNP in the majority of cases, has a neutrophilic profile, and is also referred to as non-type 2. Type 2 is IL-5 positive with a moderate eosinophilic inflammation, clinically resembling CRSwNP rather than CRSsNP. Type 3 is nearly exclusively phenotyped as CRSwNP with highly elevated levels of IgE, ECP and IL-5, representing the most severe form of CRSwNP with high prevalence of asthma comorbidity and recurrences after treatment [11]. In Asia, the inflammatory background in CRSwNP is mainly of type 1/type 17[23] with a low proportion of type 2 CRSwNP[24]. This section will focus on the type 2 inflammatory markers that is the main focus of this thesis.

2.4.1 IgE

In CRSwNP, total IgE is often highly elevated in serum and in nasal polyp tissue compared to CRSsNP and controls. IgE is produced by plasma cells upon Th 2 signals, such as IL-4 and IL-13, mediating a class switch recombination to IgE positive cells [25]; this takes place in germinal centres in secondary lymphoid tissues and also within the polyps [26]. In a subgroup of CRSwNP patients, IgE specific to staphylococcal enterotoxins (SE-IgE) is found in nasal polyp tissue and in serum[27].

2.4.2 ECP

ECP is a marker of eosinophilic inflammation. It is produced by eosinophils, stored in secondary granules within the cell and is released when eosinophils degranulate. The role of ECP in inflammation is not entirely understood, but elevated levels of ECP have been seen not only in CRSwNP, but also in other eosinophilic inflammatory diseases such as asthma[28] and atopic dermatitis[29]. ECP is thought to mirror the numbers of activated eosinophils in tissue and blood[30] and studies imply that elevated levels of ECP cause epithelial damage in the nasal mucosa [20, 30].

2.4.3 Interleukins

Th2 cells are the major source of IL-4, but in addition, mast cells, basophils and eosinophils are known to produce IL-4 [31]. IL-4 contributes to the inflammation in CRSwNP in multiple ways. It binds to the IL-4 receptor α (IL-4Rα) subunit on T-cells, present in both IL-4R type I and type II, causing them to mature from naïve T0 cells into Th2 cells. IL-4 has the capability to upregulate IgE receptors on the cell surface of mast cells, basophils, B-lymphocytes and mononuclear phagocytic cells and thus to upregulate the IgE-mediated immune response. By its interaction with vascular cell adhesion molecule-1, IL-4 directs the migration of eosinophils, basophils, T lymphocytes and monocytes to areas with inflammation and IL-4 can also prevent apoptosis of T lymphocytes [32]. IL-4 and IL-13 have structural similarities and share the same receptor, IL-4R type II, that is formed by the IL-4Rα and IL-13Rα subunits. IL-13 stimulates mucus hypersecretion, IgE production, airway hyper responsiveness and subepithelial fibrosis [33, 34]. However, it does not promote Th2 differentiation since IL-13 receptors are not expressed on T-lymphocytes [32]. IL-5 sources include cells that express the IL-5 receptor (IL-5R), basophils and eosinophils, and cells that do not express the IL-5R, such as Th2 cells, mast cells, invariant natural killer T cells and non-B/non-T cells. IL-5 takes part in promoting the proliferation and maturation of eosinophils in the bone marrow, in their migration to tissue sites and in survival of eosinophils [35]. IL-5 also causes secretion of ECP [36]. IL-5 is remarkably elevated in CRSwNP patients with non-allergic asthma and aspirin sensitivity [37]. For a schematic overview of type 2 inflammation, see figure 2.

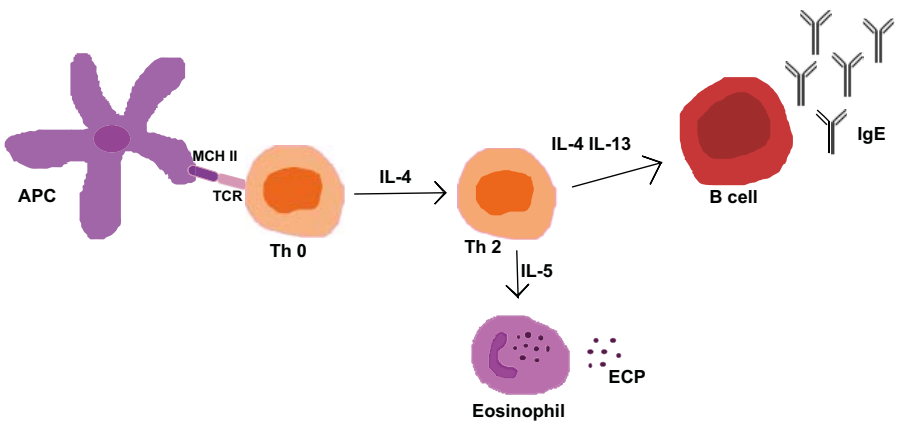


Figure 2: A schematic overview of type 2 inflammation. APC; Antigen presenting cell, MCH II; major histocompatibility complex class II, TCR; T-cell receptor, Th0; naïve T cell, IL; Interleukin, Th2; T helper cell type 2, ECP; Eosinophil Cationic Protein, IgE; Immunoglobulin E.

2.4.4 Eotaxins

Eotaxins, a family of three different chemokines, eotaxin-1 (CCL-11), eotaxin-2 (CCL-24) and eotaxin-3 (CCL-26) are released by a number of different cell types, including airway epithelial cells, when exposed to IL-4 and IL-13 [38]. Eotaxins are all potent chemotactic factors for eosinophils and are elevated in different kinds of eosinophilic diseases such as asthma, atopic dermatitis and CRSwNP [39]. The effect of the eotaxins on eosinophils are similar, they all act on the CCR-3 receptor, but their genes have different chromosomal locations [40, 41]. The CCR-3 receptor is eotaxin-specific and is expressed on eosinophils and T-lymphocytes. T-lymphocytes that express CCR-3 are involved in a number of different activities, they produce and secrete Th2-cytokines that prime and prolong survival of eosinophils [39, 42]. Eotaxins are also involved in mediating transepithelial eosinophil migration [43-45] and eotaxin-3 has been identified as the most dominant recruiter of eosinophils in asthma [46].

2.4.5 Periostin

Periostin is a member of the faciclin family, an extracellular matrix protein of 90kDa involved in the regulation of collagen deposition and fibrosis. Elevated levels of periostin have been associated with angiogenesis, re-entry into the cell cycle and with accelerated cell growth and periostin is strongly expressed in collagen rich fibrous connective tissue[47, 48]. The POSTN gene is responsible for periostin production, and the production is induced by the Th2 cytokines IL-4 and IL-13[49]. The last couple of years periostin has been suggested as a good marker of eosinophilia/Th2 inflammation [50, 51]. In asthmatic patients periostin is increased in sputum, serum and in airway epithelial cells, and is thought to play a role in airway remodelling[49, 51, 52]. Serum periostin has also been shown to predict clinical response to two types of monoclonal antibody (mAb) treatments (lebrikizumab and omalizumab), targeting the type 2 response, among patients with allergic asthma[53, 54]. A number of studies have shown elevated levels of periostin in polyp tissue from patients with CRSwNP compared to patients with CRSsNP, patients with allergic rhinitis and controls[55, 56] and an upregulation of the periostin gene in nasal polyp tissue[57]. Elevated levels of periostin in nasal polyp tissue in CRS is associated with disease severity measured by elevated Lund-Mackay scores on computed tomography (CT) scans[58, 59]. Serum and nasal periostin levels are decreased after treatment with oral glucocorticoid steroids (GCS), doxycycline, omalizumab and mepolizumab in CRSwNP patients[60].

2.5 Mucosal remodeling

In CRS, the sino-nasal mucosa is altered with changes in tissue structure and extracellular matrix protein deposition. Different patterns are seen in CRSwNP compared to CRSsNP. CRSsNP is characterized by fibrosis and goblet cell hyperplasia[61] whilst CRSwNP is characterized by formation of pseudocysts, inflammatory cell infiltrates, albumin and fibrin deposition and stromal tissue oedema within the polyps[62]. In CRSwNP, thickening of the basal membrane is related to disease severity and duration and is linked to underlying asthma and aspirin exacerbated respiratory disease (AERD) comorbidity[63]. The remodelling seen in CRSwNP is much like the one seen in asthma[62], despite the different embryonic origin of the sino-nasal and tracheal epithelium (ectoderm vs endoderm). In CRSwNP, the integrity of the epithelial barrier is compromised with a decreased expression of tight junctions and with an increased general permeability as result[64]. The underlying mechanism of remodelling is not fully understood, previously the chronic inflammation was thought to start the event but the notion that remodelling exists in early stages of the disease suggests that the remodelling occurs in parallel rather than as a result of inflammation[65].

2.6 Diagnostic tools

2.6.1 Clinical investigation

A variety of clinical diagnostic tools are available to diagnose CRS. However, the diagnosis is often made at primary health care centres based on symptoms alone. Nasal endoscopy helps phenotyping CRS into CRSwNP or CRSsNP, where polyps or swelling and/or mucopurulent discharge is easily identified. Nasal endoscopy is a standard examination that can be done with or without decongestant and local anaesthesia. CT-scans show the extent of the disease and possible anatomical details, important when planning surgery[1]. Information on comorbidities such as asthma and AERD is important in order to predict response to ordinary treatment and the likely progress of disease[66], but clinical investigation and medical history cannot alone identify different endotypes.

2.6.2 Biomarkers

Eosinophils in nasal polyp tissue is commonly used as an indicator of eosinophilic disease in nasal polyp patients. However, there is little or no consensus on cut-off values to determine tissue eosinophilia[67]. Blood eosinophilic count (EBC) is commonly used as a surrogate marker, the correlation between eosinophils in nasal polyp tissue and EBC is however not strong and changes after surgery[68] or pharmacotherapy[69]. Several different potential biomarkers have gained interest as markers of CRS in recent years, but yet there are no robust biomarkers in clinical use to endotype CRS.

2.7 Treatment

2.7.1 Current treatment

Current treatment options of CRS consist of saline irrigation and intra nasal GCS as first line therapy, short courses of per oral GCS and antibiotics as adjuvant therapy and Functional Endoscopic Sinus Surgery (FESS) in case of failure [70]. Patients who choose to undergo surgery have lower QoL scores than patients who stay on the first line therapy[71]. A group of patients with CRSwNP, especially those with asthma, AERD and patients with SE-IgE in nasal polyp tissue, experience no or little effect of traditional pharmacologic treatment or experience rapid recurrence of the disease after surgery[72]. All through the years, different types of surgical approaches of CRS have been used ranging from the least extensive "polyp extraction" to the most extensive "nasalization" procedures.[73-75]. Despite different surgical techniques the relapse rates in CRSwNP after surgery is reported to be 40-90% depending on follow-up time[9, 76, 77]. Randomised controlled surgical studies give rise to several difficulties, and therefore large reliable studies are missing.

2.7.2 Novel treatments

Given the pathophysiological similarities between CRSwNP and asthma, the new medications in asthma, mainly mAbs targeting type 2 inflammatory responses, have rendered a lot of interest also in the CRSwNP field. The results for some of the treatments are promising.

2.7.2.1 IL-4/IL-13 – Dupilumab (Dupixent®)

Targeting the IL-4 or IL-13 pathways separately, have not proven successful in asthma[32, 53]. However, since IL-4 and IL-13 have shared signalling pathways the possibility to block both these cytokines has rendered some attention. Dupilumab is a fully humanized mAb targeting the IL4Rα subunit and inhibits both IL-4 and IL-13 signalling pathways [78]. Dupilumab has proven to be effective in the treatment of asthma, improving lung function and reducing the number of exacerbations in patients with uncontrolled persistent asthma[79]. In patients with atopic dermatitis dupilumab treatment reduces the Eczema Area and Severity Index [80]. In CRSwNP patients, dupilumab reduces the nasal polyp score, it also improves smell, CT-scores, and QoL [81, 82]. Dupilumab is the first mAb approved for the treatment of CRSwNP in USA and Europe.

2.7.2.2 IgE – Omalizumab (Xolair®)

Omalizumab is a fully humanized mAb that binds to free circulating IgE via the Cε3 domain and thereby reducing free IgE by 84-99%. By blocking the binding of IgE to the high-affinity IgE receptor on mast cells and basophils, the allergen-induced

degranulation is averted and the effect of the release of inflammatory mediators and cytokines is inhibited. Omalizumab treatment also leads to a down-regulation of IgE receptors on dendritic cells, mast cells and basophils [83-85]. Omalizumab is approved for treatment of persistent moderate-to-severe asthma. Patients with CRSwNP treated with omalizumab show a significant reduction in nasal polyp score in the same magnitude as three weeks' treatment with per oral GCS, but the effect of omalizumab lasted longer and showed less side effects compared to systemic GCS therapy[86]. Omalizumab is not yet approved for the treatment of CRSwNP but is used as off-label treatment in many clinics. Two phase 3 studies regarding omalizumabs effect on CRSwNP have been completed, reports indicate that they meet primary and key secondary endpoints; but results have not yet been published (Clinical trial number NCT03280537 and NCT03280550).

2.7.2.3 IL-5 – Mepolizumab (Nucala®)

Mepolizumab is a fully humanized IgG1 mAb that binds with high affinity to free IL-5, preventing its binding to the IL-5 Receptor α expressed on eosinophils and its progenitors [87]. Several studies have shown reduction of sputum and blood eosinophils, a significant reduction of asthma exacerbations, improved QoL scores and reduced need of oral corticosteroids [87-91]. Mepolizumab is now approved for treatment of severe refractory eosinophilic asthma with EBC>300cells/microL. In 2011, a randomized placebo controlled trial was published where patients with severe nasal polyposis refractory to corticosteroid therapy received two mepolizumab or placebo injections 28 days apart. 60% of the treated patients demonstrated a significantly improved nasal polyp score and CT score. This change, along with improved smell was maintained for up to 36 weeks' post treatment, indicating a long-term effect of mepolizumab [92].One phase 3 study regarding mepolizumabs effect in CRSwNP has been completed (Clinical trial number NCT03085797) but results have so far not been published.

3 MATERIALS AND METHODS

This section contains a brief description of materials and methods used in the different studies. More information can be found in the individual papers I-V.

3.1 Human subjects

Paper I – Patients from Belgium, Sweden, Netherlands, Finland and Germany participating in the GA^{2}LEN cohort (Global Asthma and Allergy European Network) were included. Based on nasal endoscopy and medical history, they were divided into CRSwNP (n=136) and CRSsNP (n=116). In addition, 104 controls were recruited. Sino-nasal tissue and blood samples were collected during surgery.

Paper II – The Ghent database: Between May 2007 and June 2018, patients who underwent surgery in the nose and/or sinuses at the Department of Otorhinolaryngology, Ghent University Hospital, Belgium were asked to be included in a research database. Prior to surgery, clinical characteristics were recorded, and at the time of surgery tissue samples were collected. The total number of patients in the database was 1459. CRS patients with complete information on history of asthma, inhalant allergy and AERD comorbidity as well as results on tissue samples for IgE, IL-5, ECP and SE-IgE were selected from the database. In a subgroup of CRSwNP patients (n=140), EBC was also available. AERD was defined as patient reported respiratory reaction to non-steroidal anti-inflammatory drugs. Asthma was defined as patient reported use of asthma medication. Allergy was defined as patient reported symptoms of inhalant allergy.

Paper III – A randomized placebo controlled phase II study where 60 patients with CRSwNP were included[81]. The study was conducted at 13 sites in US and Europe (Belgium, Sweden, Spain) between August 2013 and August 2014. Eligible patients were aged 18-65 years and had symptoms of CRS despite treatment with intra nasal GCS spray and a nasal polyp score of at least 5 (at least 2 per nostril). Patients received dupilumab (N=30) or placebo (N=30) subcutaneously once a week during 16 weeks. Nasal secretions were collected at inclusion and every 4 weeks, and in a subgroup of patients (n=12) nasal polyp tissue was obtained.

Paper IV – CRSwNP patients (n=84) who underwent sinus surgery for nasal polyps between January 2015 – August 2016 were reviewed. Patients lost to follow-up within seven months after surgery were excluded. In total 50 patients were included in this study. Patients who underwent a standard FESS with the minimal invasive mucosa sparing technique were considered controls and called non-reboot (n=20), while patients who underwent the reboot technique were considered cases (n=30) and subdivided into two groups; full reboot (with Draf III) (n=12) and partial

reboot (without Draf III) (n=18). SNOT-22 were sent out to all study subjects by mail in January 2018 and in March 2018. All patients were given the same post-operative care and follow-up.

Paper V – Patients aged 18 years or older with symptomatic bilateral severe CRSwNP scheduled for sinus surgery at the Department of Otorhinolaryngology, Ghent University Hospital, Belgium were included. Nasal polyps and affected mucosa from the different sinuses, and biopsies from the middle turbinate were collected, in total 76 samples from 11 patients. Inferior turbinates from healthy patients undergoing rhino-septoplasty was used as controls. 21 patients who underwent re-boot surgery were followed-up during 12 months post-operatively. At surgery, nasal polyp biopsies, serum and nasal secretions were collected and at 12 months' follow-up nasal secretions and serum were collected. Nasal secretions and serum samples from healthy patients (n=13) participating in the GA^{2}LEN cohort were used as controls.

Nasal polyp score (paper III, IV, V) – 4 points on each side, maximum 8 points.

Polyp score	Polyp size
0	No polyps
1	Small polyps in the middle meatus not reaching below the middle turbinate
2	Polyps reaching below the lower border of the middle turbinate
3	Large polyps reaching the lower border of the inferior turbinate or polyps medial to the middle turbinate
4	Large polyps causing complete obstruction of the inferior nasal cavity

Polyp recurrence (paper IV, V) – nasal polyp score of at least one on either side. *Exclusion criteria* (paper I-II, IV-V) – patients with other disorders, such as cystic fibrosis, primary ciliary dyskinesia or Eosinophil granulomatosis with polyangiitis (EGPA) and patients participating in other studies of monoclonal antibody treatment were excluded. *Reboot technique* (paper IV, V*)* – endoscopic sinus surgery including clearing all polyps and sinus mucosa down to the periosteum from all sinuses. With or without Draf III, depending on involvement of the frontal sinus. The inferior, and when possible, the middle turbinate is preserved.

Draf III (paper IV-V) – a surgical technique creating a wide, joint, opening to the frontal sinuses by resection of the frontal sinus floor and superior nasal septum[93]. *Preoperative treatment* (paper I, II, IV, V) – all patients were asked to stop intra nasal and per oral GCS treatment two and four weeks prior to surgery. *Postoperative treatment* (paper IV, V) – Nasal douching, doxycycline 100mg per day for three months and topical GCS drops containing fluticasone propionate. *Location* (paper II, IV, V) – all patients underwent surgery at the Department of Otorhinolaryngology at Ghent University Hospital in Belgium. *Ethical approval* (paper I-V) – All studies were approved from ethical committees at respective study site and all patients signed informed consent prior to inclusion. *SNOT-22* (paper IV) – disease specific symptom score. 22 questions scored 0-5, maximal score 110. Higher scores represent worse symptoms[94, 95].

3.2 Sino-nasal tissue (paper I-V)

Sino-nasal tissue was obtained during scheduled surgery (paper I, II, IV, V). In paper III nasal polyp biopsies were obtained at the open patient clinic after topical application of local anesthesia (nafazoline 0.17 mg/ml and lidocainhydrochloride 34 mg/ml) for 15 minutes carefully making sure that the polyp score was not altered. All tissue was snap frozen in liquid nitrogen and stored at –80º C until analysis. Snap-frozen tissue samples were homogenized and disrupted at 50 Hz for 2 minutes with the Tissue Lyser LT (Qiagen Benelux, Antwerp, Belgium) and 1 ml 0.9% NaCl with Complete, an EDTA-free protease inhibitor (Roche Diagnostics Belgium, Vilvoorde, Belgium), was added per 0.1 g of tissue[27, 96]. In paper IV type 2 inflammation in tissue was defined as IL-5 > 12.98pg/g and SE-IgE positivity in tissue was defined as SE-IgE > 3.85kUA/g[11].

3.3 Nasal secretions (paper III, V)

Nasal secretions were obtained by inserting Post-operative sinus packings (3.5 cm IVALON®, Fabco®, New London, CT, USA) into the nasal cavities and left for 5 minutes. 3 mL of saline (0.9% NaCl) was added to each of the tubes and they were then incubated for 2 hours. The tubes were centrifuged at 1500xG for 15 minutes at 4º C[97]. In paper III the analyzed nasal secretions were reported without further normalization, in paper V the results were reported after normalization to weight.

3.4 Blood samples (paper I, II, V)

In paper II, blood samples were collected at the open patient clinic and analysed for total eosinophilic count according to regular guidelines at the laboratory at Ghent University Hospital. In paper I and IV, blood samples were collected routinely prior to surgery and in paper V also at twelve months' follow-up. The serum was stored in -25º C until analysis.

3.5 Antibody assays

3.5.1 Periostin (Paper I)

Serum periostin was measured using the clinical trial version of the Elecsys® Periostin assay on the e 601 module of the cobas 6000 system[98]. The Elecsys® Periostin assay is a fully automated assay similar to an ELISA. A sandwich complex is formed between periostin, a biotinylated antibody and a ruthenylated antibody, and is captured on the surface of added streptavidin-coated micro particles. The amount of captured complex and therefore the periostin level in the sample is measured using electrochemiluminescence technology. The two antibodies that are used target different epitopes of periostin allowing a more specific measurement.

3.5.2 Enzyme-linked immunosorbent assay – ELISA (paper III)

ELISA is a method for detection and quantification of an antibody or antigen in a sample. A sandwich-ELISA (Quantikine ELISA Kit®, R&D Systems, Minneapolis, MN, USA) was used for quantification of eotaxin -3 in saline eluates from nasal swabs. The ELISA used was based on a microplate pre-coated with antibodies against the antigen of interest. When adding a sample to the microplate, potential antigens in the sample bind to the antibodies. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for the antigen of interest is added and after adding a substrate solution, a color develops which is proportional to the amount of bound antigen.

3.5.3 ImmunoCAP fluorescent enzyme immunoassay (paper I-V)

Different types of ImmunoCAP techniques were used to assess IgE, ECP and a mixture of SE-IgE (staphylococcal enterotoxins A (SEA) and C (SEC) and toxic shock syndrome toxin 1 (TSST-1)) in serum, nasal secretion and sino-nasal tissue homogenates in paper I-V. ImmunoCAP is a method used for the quantification of an antibody similar to an ordinary sandwich ELISA but where the reaction takes place in a solid phase. An antigen is covalently coupled to the solid phase and binds to the antibody of interest. After washing, enzyme labeled antibodies against the marker are added to form a complex. Following incubation, the unbound enzyme-anti-marker is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured. The fluorescence is directly proportional to the concentration of the marker in the sample. The higher the response, the more marker is present in the sample. The UniCAP system is a predecessor of ImmunoCAP. In paper I-V the UniCAP system (Phadia, Uppsala, Sweden/ Thermo Fisher Scientific, Phadia, Grootbijgaarden, Belgium) was used. In paper II the ImmunoCAP (Phadia AB, Uppsala, Sweden) was used for assessing biomarkers in nasal secretions.

3.5.4 Luminex (paper I-V)

Luminex immunoassay was used for measuring multiple cytokines[99], for example interleukins and chemokines, in serum samples, nasal secretion and sino-nasal tissue homogenates. Commercially available kits (Luminex Performance Assay/ Screening Human assay) were used and measured on a Bio-Plex 200 Platform (Bio-Rad Laboratories Temse, Belgium). Luminex is a technology in which several proteins or peptides in one sample can be quantified. The method is similar to sandwich-ELISA, with the exception that magnetic beads dyed with fluorescent dyes are covalently coupled to the antibodies directed against the antigen of interest. Each bead thus has a distinct colour code permitting discrimination of individual antigens. Generally, Luminex has lower detection limits than an ELISA and is more time efficient but can be more expensive. Another advantage is that only a small sample volume is needed.

3.6 Statistical methods (paper I-V)

Graph Pad Prism version 7 and 8 for Mac OS X (GraphPad Software, Inc., La Jolla, CA) was used for all statistics, except in the prediction model in paper II and for calculations of nasal secretions in paper III where STATA Statistical Software, version 13.1 (Stata Corp, College Station, Texas) was used and in paper IV where Statistical Package for Social Sciences, version 25.0 (SPSS, Chicago, IL) was used for the Kaplan-Maier curves. Non-parametric tests were used since the data were not normally distributed. A p-value < 0.05 was considered significant. Mann-Whitney (paper I, II, IV) was used to compare median levels between two groups. The Kruskal-Wallis test with Dunn's multiple comparison (paper I, IV, V) was used to assess differences in median levels between more than two groups. The Wilcoxon test (paper III, IV) was used for paired analysis. Spearmen correlation test (paper I, II) was used to assess correlations, and the results were interpreted as suggested by Hinkle et al[100] (R=0.9-1 very high positive, R=0.7-0.9 high positive, R=0.5-0.7 moderate positive, R=0.3-0.5 low positive, R=0.0-0.3 negligible). Chi^2-test (paper II) or Fisher's exact test (paper I-V) was used to compare proportions[101]. ROC-curves (paper I) were used to determine best cut-off between serum periostin, IgE and SE-IgE and IL-5 and SE-IgE positivity in tissue. For biomarkers in nasal secretions in paper III, the areas under the concentration versus time curves from time of first treatment to week 16 (AUC_{0-16}) were estimated by trapezoidal analysis. The comparison of treatment effects from the mixed-effect model with repeated measures analyses were based on the LS mean change (with 95% confidence intervals and *P* values) from baseline to Week 16.

4 RESULTS AND COMMENTS

4.1 Inflammation in severe CRSwNP (paper V)

Our aim was to understand whether inflammation in severe type 2 CRSwNP was limited to the polyps or if the non-polypoid sinus mucosa also contributed. 11 patients scheduled for reboot surgery at Ghent University Hospital, Belgium, were included. During surgery, polyps and non-polypoid mucosa from the different sinuses, polyps from the nose and middle turbinate tissue were obtained, in total 76 samples. 16 inferior turbinates from healthy patients were used as controls. Polyps and non-polypoid mucosa from all the sinuses and polyps in the nose expressed elevated levels of IgE, ECP and IL-5 compared to control tissue. The middle turbinate expressed elevated levels of IgE, ECP but not IL-5. (Figure 3)

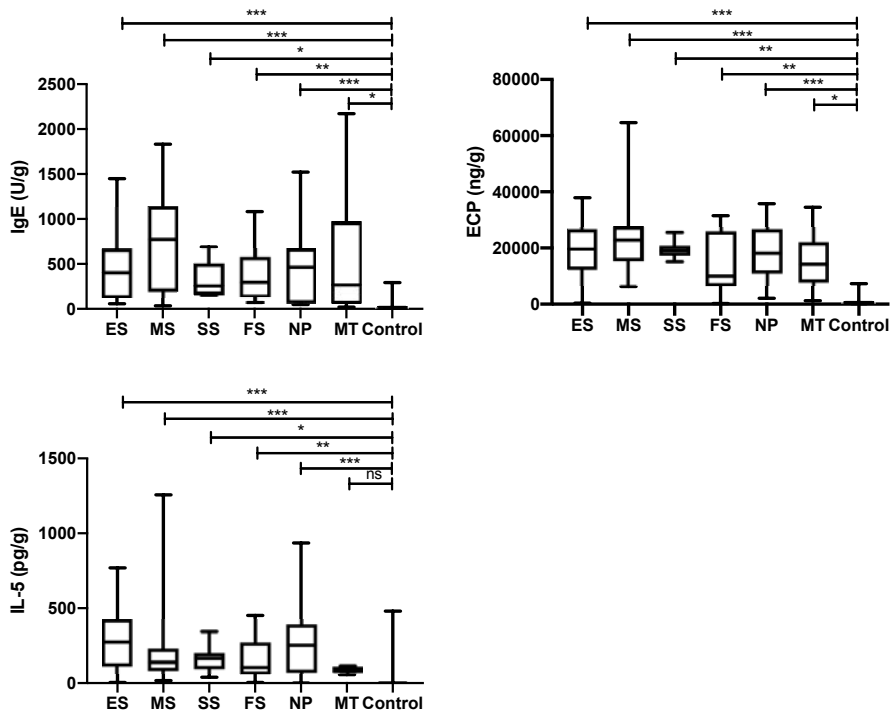


Figure 3: Concentrations of IgE, ECP and IL-5 in tissues from the different locations. The boxes indicate the median and IQR, error bars min;max; Kruskal-Wallis test with Dunn's multiple comparison; *** $p \leq 0.001$, ** $p \leq 0.01$, * $p < 0.05$, ns; not significant. ES; ethmoid sinus, MS; maxillary sinus, SS; sphenoid sinus, FS; frontal sinus, NP; nasal polyp, MT; middle turbinate.

Polyps and non-polypoid mucosa expressed elevated levels of IgE, ECP and IL-5 compared to controls, no difference was seen between polyps and non-polypoid mucosa. (Figure 4)

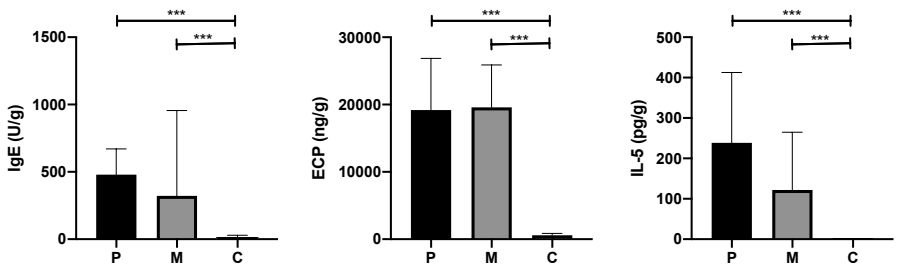


Figure 4: Concentrations of IgE, ECP and IL-5 in polyps (P), non-polypoid mucosa (M) and control tissue (C). The boxes indicate the median and IQR; Kruskal-Wallis test with Dunn's multiple comparison; *** $p \leq 0.001$, ** $p \leq 0.01$, * $p < 0.05$

4.1.1 Comments

In severe type 2 CRSwNP, all sinuses expressed elevated levels of type 2 inflammatory markers compared to controls. The inflammation was not limited to the actual polyps but was equally present in non-polypoid sinus mucosa.

4.2 Type 2-shift in CRS (paper II)

There are wide geographical and phenotypical differences with the regard to inflammatory background in CRS[24]. In Asia, a shift from type 1 towards type 2 inflammation in CRSwNP has been described[102]. We aimed to investigate whether this shift can be seen in CRS in Western Europe as well.

4.2.1 CRSwNP

CRSwNP patients who underwent surgery at University Hospital in Ghent during two different time periods (2007-2010 and 2016-2018) were compared. The two cohorts were equal regarding gender, age and comorbidities. The proportion of patients who had previous sinus surgery were not different. In the CRSwNP patients (n=102 and n=90), an increase in tissue levels of IgE, ECP and IL-5 was seen in the later cohort compared to the earlier. When stratified for comorbidities, the increase could only be seen in non-asthmatic, non-allergic patients (figure 5), among these, a higher proportion of patients also had IL-5 positive polyps (63.4% vs 86.7% p=0.033). There was no difference in EBC between

the two cohorts (median (IQR) 2007-2010 293.5(162.0;574.8) 2016-2018 420.0 (235.0;747.5 p=0.147)). Also, in this group, there were no differences in comorbidities or proportions of patients with a history of previous surgery.

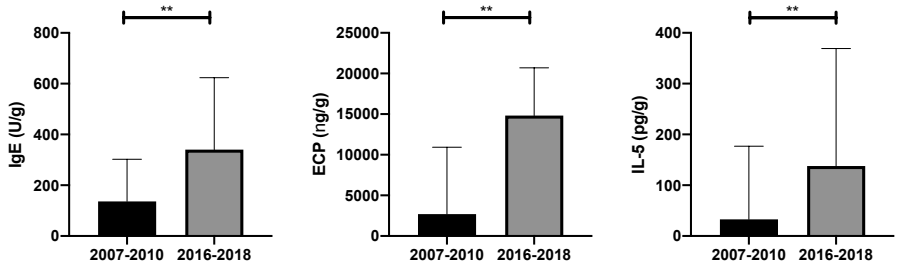


Figure 5: IgE, ECP and IL-5 in patients without asthma/allergy. Levels presented as median and IQR. ** p≤0.01, Mann-Whitney.

4.2.2 CRSsNP

CRSsNP patients who underwent sinus surgery at the University Hospital in Ghent during two different time periods (2007-2010 and 2015-2018) were compared. The two groups were similar in regard to comorbidities. In CRSsNP patients (n=39 and n=26) ECP and IL-5 were elevated in the later cohort compared to the earlier (figure 6). This increase was, as for the CRSwNP patients, only seen in the non-asthmatic group (n=31 vs n=18). In the proportion of patients who were positive for IL-5 or SE-IgE in nasal polyp tissue, no differences were seen.

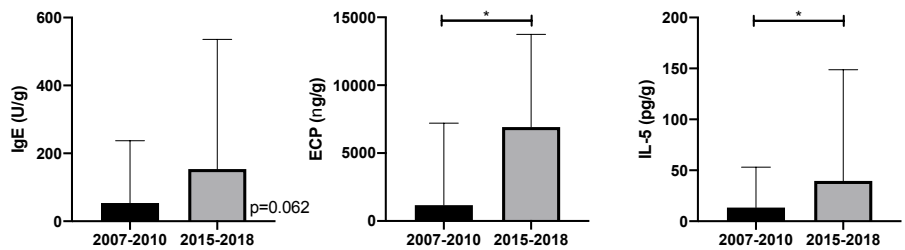


Figure 6: Levels of IgE, ECP and IL-5 in CRSsNP tissue. Levels presented as median and IQR. * p<0.05. Mann-Whitney.

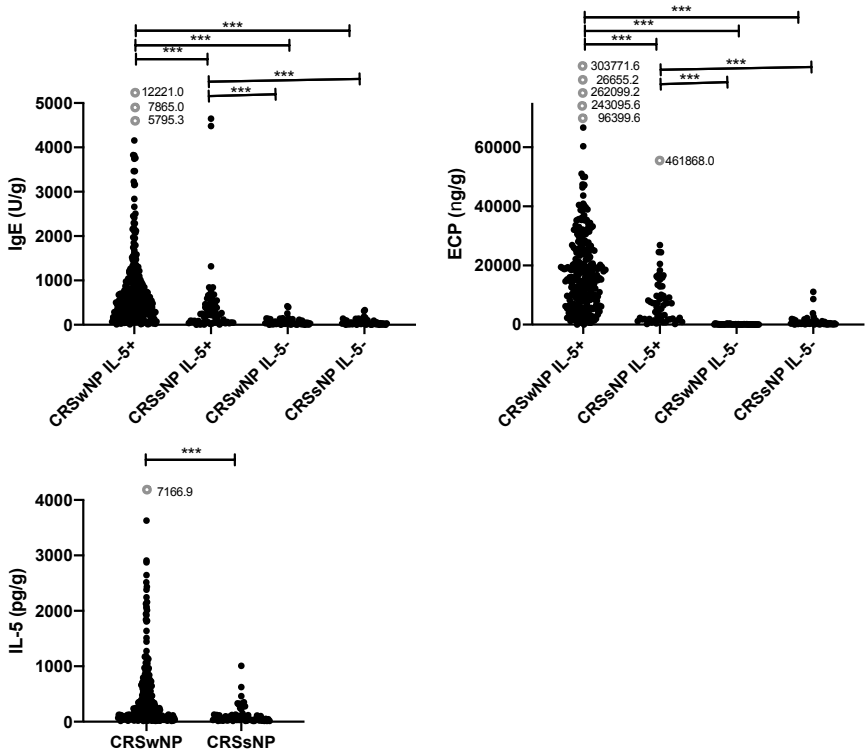
4.2.3 Comments

Our data suggests an actual shift of endotype in CRSwNP patients over time, with elevated levels of IgE, ECP and IL-5, and an increase of the proportion of IL-5 positive patients, much like the shift seen in parts of Asia[102-104]. Interestingly, the shift was only seen in non-asthmatic patients. This shift, towards a type 2 endotype, also seemed to be happening in CRSsNP patients, although not as pronounced, also here only observed in non-asthmatic patients. However, due to a small cohort of asthmatic patients these results must be interpreted with some caution. The results could be due to a selection bias. Using asthma and AERD comorbidities and the proportion of patients who had undergone previous surgery as a marker of disease severity, no differences were seen between the cohorts, and the shift was only present in non-asthmatic patients, i.e. patients with a mild form of disease.

4.3 Endotypes and phenotypes; effect on type 2 inflammatory markers (paper II)

4.3.1 IL-5 positive and negative CRS

438 patients with CRS (CRSwNP n=323, CRSsNP n=115) participating in a research database in Ghent, Belgium, were included. Patients were stratified on phenotype i.e. presence or absence of polyps. IL-5 positive patients, regardless of phenotype, expressed elevated levels of IgE and ECP compared to IL-5 negative patients. In the IL-5 positive group there was a significant difference in comorbidities (asthma, 45.6% vs 30.0% p=0.031, AERD 19.9% vs 5.0% p=0.004) and in SE-IgE positivity in polyps (45.2% vs 26.7% p=0.009) between CRSwNP and CRSsNP, allergy did not differ between the groups. This difference was not seen in the IL-5 negative group (figure 7).



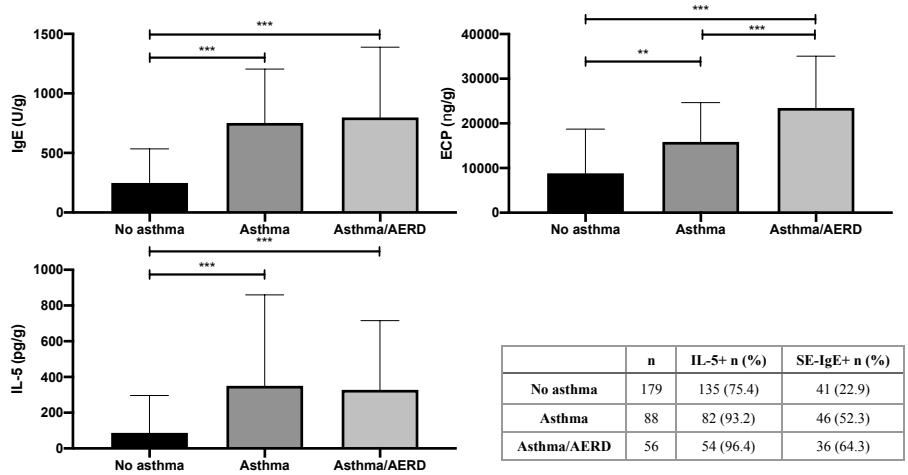
IL-5 +	CRSwNP	CRSsNP	p-value	IL-5 -	CRSwNP	CRSsNP	p-value
n	272	60		**n**	51	55	
Asthma n (%)	124 (45.6)	18 (30.0)	0.031*	**Asthma n (%)**	8 (15.7)	9 (16.3)	>0.999
Allergy n (%)	123 (45.2)	24 (40.0)	0.474	**Allergy n (%)**	11 (21.6)	12 (21.8)	>0.999
AERD n (%)	54 (19.9)	3 (5.0)	0.004*	**AERD n (%)**	2 (3.9)	1 (1.8)	0.607
SE-IgE + n (%)	123 (45.2)	16 (26.7)	0.009*	**SE-IgE + n (%)**	1 (2.0)	0 (0)	0.481

Figure 7: Levels of IgE and ECP in IL-5 positive (IL-5+) and IL-5 negative (IL-5-) in CRSwNP and CRSsNP patients and levels of IL-5 in IL-5 positive CRSwNP and CRSsNP patients. Dots are individual values. Circles in grey indicate individual values above the maximal range of the y-axis. *** $p \leq 0.001$. Numbers and proportion of patients with asthma, allergy, AERD and SE-IgE positivity in polyps in the different groups, significant numbers indicated with *. Kruskal-Wallis test with Dunn's multiple comparison, Mann-Whitney, and Fisher's exact test.

4.3.2 Comorbidities impact on type 2 markers in CRSwNP

323 CRSwNP patients were stratified based on asthma and/or AERD comorbidity. CRSwNP patients with asthma and/or AERD expressed elevated levels of IgE, ECP and IL-5 compared to CRSwNP patients without asthma and/or AERD. AERD

comorbidity did not enhance levels of IgE or IL-5 compared to asthma only, ECP was elevated in AERD compared to asthma (figure 8).



	n	IL-5+ n (%)	SE-IgE+ n (%)
No asthma	179	135 (75.4)	41 (22.9)
Asthma	88	82 (93.2)	46 (52.3)
Asthma/AERD	56	54 (96.4)	36 (64.3)

Figure 8: IgE, ECP and IL-5 in patients without asthma or AERD, with asthma only, with asthma and AERD. Mutually exclusive groups. Levels presented as median and IQR. *** $p \leq 0.001$, ** $p \leq 0.01$. Kruskal-Wallis test with Dunn's multiple comparison. Number and proportion of IL-5 and SE-IgE positivity in polyps.

4.3.3 Comments

Within the CRS group, two different types could be identified, IL-5 positive and IL-5 negative patients based on inflammatory levels in sino-nasal polyps. IL-5 negative patients seemed to be more coherent, in terms of inflammatory patterns and comorbidities, regardless of endotype, compared to IL-5 positive patients where the CRSwNP patients had elevated levels of inflammatory markers and type 2 related comorbidities. Levels of type 2 inflammatory parameters were not directly linked to polyp formation. CRSwNP patients with asthma and/or AERD expressed elevated levels of IgE, ECP and IL-5. AERD did not seem to enhance the levels of IgE and IL-5, suggesting that asthma itself can cause maximal type 2 inflammation. However, ECP is elevated in AERD, as seen in previous studies[105].

4.4 Identifying type 2 inflammation in CRSwNP; clinical markers and biomarkers (paper I and II)

It has been difficult to identify biomarkers and clinical signs to properly endotype CRS, which is crucial in order to implement a personalized approach to CRS treatment. We therefore explored a combination of novel and traditional biomarkers and comorbidities to identify type 2 inflammation in sino-nasal polyps.

4.4.1 Serum periostin, IgE and SE-IgE (paper I)

377 patients participating in the GA^{2}LEN cohort were included. Based on history and nasal endoscopy they were divided into CRSwNP (n=144), CRSsNP (n=123) and controls (n=110). Serum and tissue samples from these patients were analysed for type 2 inflammatory markers. Serum levels of periostin were elevated in patients with CRSwNP compared to CRSsNP and controls (figure: 9).

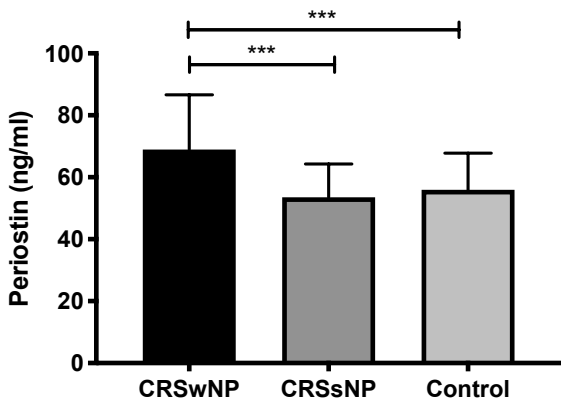


Figure 9: Serum periostin in CRSwNP, CRSsNP and controls. Median and IQR. Kruskal-Wallis with Dunns' multiple comparison. *** $p \leq 0.001$.

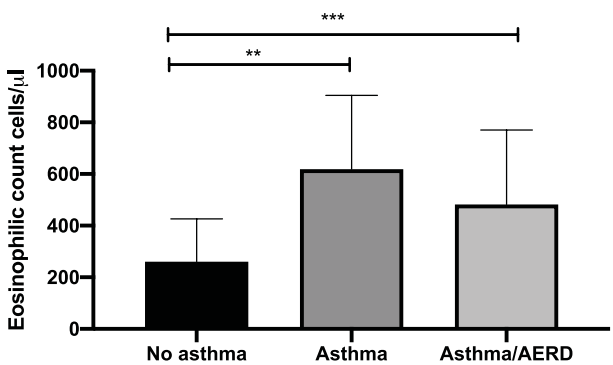
ROC analyses were performed to see whether serum markers could predict presence of IL-5 and/or SE-IgE in polyp tissue in CRSwNP patients. 108 CRSwNP patients had a full dataset for serum markers, tissue IL-5 and tissue SE-IgE. 85.2% of these patients were IL-5 positive in nasal polyp tissue. Serum periostin > 48.5ng/ml predicted for IL-5 positivity with a sensitivity of 93.5% and a specificity of 63.5% ($p<0.0001$). 20.4% of the patients were SE-IgE positive in nasal polyp tissue, all of them had serum periost in 48.5ng/ml. Among these patients (n=92), serum IgE > 96kUA/l and serum SE-IgE > 28kUA/l predicted for SE-IgE positivity with a sensitivity of 77.3% and a specificity of 87.1% ($p<0.0001$) (table 1).

N=108	Cut-off level (proportion positive)	AUC	Sensitivity	Specificity	p-value
Tissue IL-5 positive (n=92)					
serum Periostin	48.5 ng/ml (86/92)	0.78	93.5%	62.5%	<0.0001
N=92					
Tissue SE-IgE positive (n=22)					
serum IgE	96 kUA/l (19/22)	0.8	86.4%	67.7%	<0.0001
serum SE-IgE	0.28 kUA/l (17/22)	0.8	77.3%	75.7%	<0.0001
Serum IgE, SE-IgE	(17/22)		77.3%	87.1%	<0.0001

Table 1: Cut-off concentrations, proportions of positive patients, area under the curve and sensitivity, specificity and p values for predicting IL-5 and SE-IgE in tissue using serum periostin, IgE and SE-IgE. P-value calculated with Fisher's exact test.

4.4.2 Blood eosinophil count and comorbidities (paper II)

140 patients with CRSwNP who underwent sinus surgery at Ghent University Hospital was included. Comorbidities, EBC and type 2 inflammatory markers in tissue were assessed. EBC was elevated in patients with asthma and/or AERD compared to CRSwNP patients without asthma/AERD (figure 10).



	No asthma/AERD	Asthma	Asthma/AERD
N	81	32	27
Eosinophilic count	260.0 (142.5;426.0)	618.5. (375.0;904.8)	482.0 (279.0;770.0)

Figure 10: Blood eosinophilic count in patients without asthma/AERD, with asthma only and with asthma and/or AERD. Levels presented as median and IQR. *** $p<0.001$, ** $p\leq0.01$, * $p<0.05$. Kruskal-Wallis test with Dunn's multiple comparison.

There were rather weak positive correlations between EBC and tissue inflammatory markers, tissue IgE (R=0.415, p<0.001), SE-IgE (R=0.214, p=0.011) ECP (R=0.410, p<0.001) and IL-5 (R=0.489, p<0.001). A prediction model based on chi^2-test (or Fisher's exact test when appropriate) was developed to investigate if any of the clinical markers, asthma, AERD and allergy comorbidity and EBC > 300 cells/microL could be used to identify patients with or without IL-5 positivity and SE-IgE positivity in nasal polyp tissue. In the total group, the likelihood of IL-5 positivity was 84.3%. Among patients with asthma or AERD, 94.2% were IL-5 positive (p<0.001). In patients without asthma/AERD the likelihood was 68.5%, but with an EBC>300cells/microL the likelihood increased to 95.2% (p<0.001). Regarding SE-IgE, 40.7% of the patients were positive. The likelihood of SE-IgE in tissue among patients with asthma/AERD was 48.8% (p=0.014). The group with asthma/AERD and EBC> 300 cells/microL had a higher proportion of SE-IgE positivity in nasal polyp tissue compared to the group without asthma/AERD and EBC<300cells/microL (p=0.002, 57.9% vs 21.9%). (Figure 11).

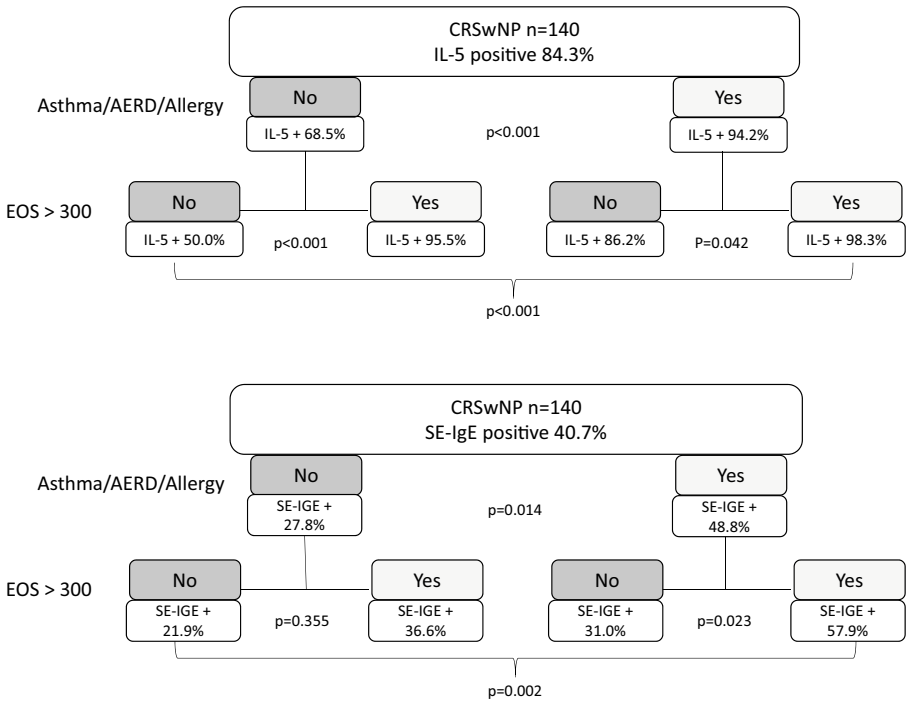


Figure 11: A prediction model for tissue IL-5 positivity and tissue SE-IgE positivity based on clinical markers, asthma and/or AERD and/or allergy comorbidity and elevated blood eosinophils (EBC > 300 cells/microL). P-values obtained by Chi^2–test or Fisher's exact test.

4.4.3 Comments

Serum periostin was elevated in CRSwNP patients compared to CRSsNP patients and controls, and could, together with serum IgE and SE-IgE, be used with reasonable sensitivity and specificity to identify type 2 CRSwNP. Tissue eosinophilia is commonly used to diagnose eosinophilic CRSwNP, however there is little or no consensus on cut-off levels of meaningful eosinophilia[67]. EBC has been suggested as a surrogate marker, in our results however, there was a weak or no correlation between EBC and type 2 inflammatory markers in polyp tissue. EBC can, together with questions about comorbidities relevant for type 2 disease, help identify CRSwNP patients with tissue IL-5 expression.

4.5 Novel treatments (paper III-V)

CRSwNP patients experienced relapse after surgery in 40-90% of cases, depending on follow-up time[9, 76, 77]. Therefore, new treatment strategies for this patient group are required.

4.5.1 Dupilumab – effect on local inflammatory markers (paper III)

In recent years, studies on mAbs, targeting different aspects of the type 2 inflammatory response have been carried out with promising results in CRSwNP patients for some therapies[86, 92] with reduction of nasal polyp score and an improvement of symptom scores.

We performed a post-hoc analysis of a randomized, double-blind, placebo-controlled phase II study regarding treatment effects of dupilumab[81] in CRSwNP patients. 60 patients with CRSwNP were included in this study. Patients received either a 600mg loading dose of dupilumab followed by 16 weeks of 300 mg dupilumab or matched placebo. All patients were on mometasone furoate nasal spray (100mg/nostril twice daily) 4 weeks prior to inclusion and continued with this throughout the study. Nasal secretions were obtained at inclusion and every 4 weeks of the study, nasal biopsies were obtained in a subgroup of patients (n=12) at inclusion and at the end of treatment. There was a decrease in total IgE and eotaxin-3 in nasal secretions in the dupilumab group compared to placebo (LS mean $AUC_{0\text{-}16}$ [±SE]; total IgE vs placebo (−7.90 [1.9] vs −1.86 [2.1] IU/ml; p=0.022): eotaxin-3 −30.06 [5.9] vs −0.86 [6.6] pg/ml; p<0.001). In nasal polyp tissue a decrease of total IgE (p=0.023) ECP (p=0.008), eotaxin-2 (p=0.008), eotaxin-3 (p=0.031), IL-13 (p=0.031) and pulmonary and activation-regulatory cytokine (PARC) (p=0.016) was observed from inclusion to end of treatment in the dupilumab group, no changes were found in the placebo group (table 2).

Change from inclusion	Dupilumab	p-value	Placebo	p-value
IgE U/g median (IQR)	-139.2 (-384.7;-36.0)	0.023*	-97.6 (312.3;350.9)	>0.999
ECP ng/g median (IQR)	-7310.0 (-9591.0;-5129.0)	0.008*	-665.0 (-2855;10393.0)	>0.999
Eotaxin 2 pg/g median (IQR)	-4117.0 (-23163.0;-1271.0)	0.008*	833.1 (-5938.0;27540.0)	0.875
Eotaxin 3 pg/g median (IQR)	-394.6 (-1892.0;-87.7)	0.031*	-206.0 (-480.7;1583.0)	0.875
IL-13 pg/g median (IQR)	-171.7 (-874.9;27.4)	0.031*	-888.1 (-1928.0;-104.2)	0.250
PARC pg/g median (IQR)	-320074.0 (-893531.0;-85552.0)	0.016*	179289 (1041;1012327)	0.250

Table 2: Changes in inflammatory markers in nasal polyp tissue from inclusion to end of treatment in the dupilumab and placebo group. Levels presented in median and IQR. Wilcoxon test. Significant numbers are indicated with*.

4.5.2 Reboot surgery (paper IV and V)

Reboot surgery, is an extensive form of endoscopic sinus surgery, involving complete removal of sinus mucosa down to the periosteum, with (full reboot) or without (partial reboot) a wide opening to the frontal sinuses (Draf III approach). In this way, the inflammatory burden is removed and the nasal mucosa can regenerate. Reboot surgery has been proposed to be more efficient in preventing relapse.

4.5.3 Recurrence and symptom scores (paper IV)

82 patients who underwent endoscopic sinus surgery due to CRSwNP at the University Hospital in Ghent, Belgium, between 2015-2107 was reviewed. Patients who were lost to follow-up within 7 months' post-surgery, and patients with congenital disorders such as cystic fibrosis were excluded. 50 patients remained and were included. 12 patients had undergone full reboot, i.e. including a Draf III procedure, 18 patients had a partial reboot, i.e. without a Draf III procedure and 20 patients had a conventional mucosa sparing procedure (non-reboot). Baseline data, including asthma and AERD comorbidity, prior surgeries and nasal polyp score did not differ between the three groups. Type 2 inflammatory markers in nasal polyp tissue and serum did not differ between the groups. The reboot group had less relapses during follow-up (longest follow-up 30 months' post-surgery) (relapse defined by a nasal polyp score of at least 1 on either side) (45% vs 13.3% p=0.02). A survival analysis showed superior results for the full reboot group, concerning cases with relapse and time to recurrence, followed by the partial reboot and the non-reboot groups (table 3).

	Non-reboot	Partial reboot	Full reboot	p-value
n	20	18	12	
Recurrence n (%)	9 (45)	3 (16.7)	1 (8.3)	0.038*◎
Earliest time for recurrence (months)	2	4	12	
	Non-reboot	**All reboot**		
n	20	30		
Recurrence n (%)	9 (45)	4 (13.3)		0.02*^

Table 3: Number and proportion of recurrence in non-reboot, partial reboot and full reboot; time to recurrence. Number and proportion in non-reboot and all reboot. ◎Chi^2-test, ^Fischer's exact test.

Despite a more extensive surgery, patients in the reboot group had significantly better SNOT-22 results 2 years after surgery compared to the non-reboot group (figure 12).

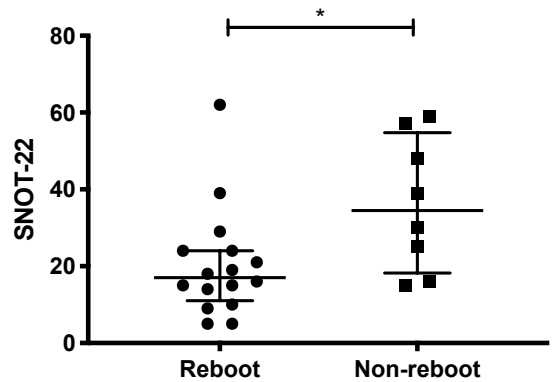


Figure 12: SNOT-22 scores of reboot and non-reboot patients 2 years after surgery. * p<0.05. Mann-Whitney.

4.5.4 Reboot; effect on local and systemic inflammatory markers (paper V)

21 patients scheduled for reboot surgery at Ghent University Hospital, Belgium, were included. 52% had comorbid asthma, 10 % had comorbid AERD and 71% of the patients had had at least 1 prior surgery. Nasal secretions and serum were collected prior to surgery and at 12 months' follow-up. Patients participating in the GA^2LEN cohort was used as controls (n=13). Type 2 inflammatory markers (IgE, ECP and IL-5) in nasal secretions were elevated at inclusion in CRSwNP patients compared to controls. In CRSwNP patients a marked decrease was seen for IgE, ECP and IL-5 from inclusion to the end of the study but did not reach the levels of the controls (table 4).

Nasal secretions	Inclusion	12 months	Controls	p-value^	p-value^	p-value¨
N	21	21	13	B vs C	12 m vs C	B vs 12 m
IgE kU/L median (IQR)	53.3 (8.8;185.8)	20.6 (4.9;96.11)	2.8 (1.7;4.7)	0.0002*	0.006*	0.03*
ECP μg/L median (IQR)	1030 (182.3;1650)	482.1 (126;1288)	42.4 (24.2;243.7)	0.002*	0.04*	0.04*
IL-5 pg/ ml median (IQR)	33.7 (2.4;309.2)	5.1 (2.1;62.6)	not detectable	-	-	0.04*

Table 4: IgE, ECP and IL-5 in nasal secretions at inclusion and after 12 months compared to healthy controls. P-value obtained by Kruskal-Wallis test with Dunns multiple comparison (^) and Wilcoxon matched pair signed rank test (¨). Significant p-values are indicated with * B = baseline, C = control, 12m = 12 months.

Serum samples were available from 19 patients at both inclusion and at a 12 months' follow-up. No decrease in serum IgE, SE-IgE or ECP was seen. In the disease-specific SNOT-22 and the health related QoL questionnaire RAND-36, both the physical component scale (PCS) and the mental component scale (MCS), were improved 12 months after surgery compared to baseline (SNOT-22 p=0.003, RAND-36 PCS p=0.03, RAND-36 MCS p=0.046). Out of the 21 patients, three patients had a unilateral nasal polyp score of one twelve months after surgery and one patient had a bilateral polyp score of two. These polyps could be removed in an open clinic setting under local anaesthesia. All patients had completely fine mucosa covering the sinus walls within 4-6 weeks and no patients suffered from major complications during surgery or postoperatively.

4.5.5 Comments

The phase II study that paper III was based on showed a decrease in nasal polyp score and a rapid improvement of smell in the dupilumab group compared to placebo and a reduction of total IgE and eotaxin-3 in serum[81]. Our data suggest, that blocking the IL-4/IL-13 pathway affects mucosal IgE production and has an effect on eosinophilic activation, survival and migration. The reduction of ECP in nasal polyp tissue suggests a local reduction of activated eosinophils. The reduction of eosinophils could not be determined due to scant polyp material. Eotaxins are highly chemotactic to eosinophils and other inflammatory cells, and are known to prolong eosinophilic survival[39, 42], mediate eosinophilic activation and direct transepithelial migration[43, 44]. A potential mechanism for reducing eosinophilic activation and infiltration in tissue, demonstrated by the reduction of eotaxins and ECP in nasal polyp mucosa and nasal secretions, may be a change in the chemotactic

gradient signaling eosinophilic migration from the blood stream into the tissue, which is in line with the rapid increase seen in blood eosinophils shortly after initiating dupilumab treatment[81]. The small number of patients available for polyp tissue analysis and the fact that all patients included were from Europe or USA are drawbacks of this study, our data is however coherent with a larger dupilumab phase III study recently published[82]. Reboot surgery reduces inflammatory markers in nasal secretion in the same magnitude as treatment with dupilumab, and the reduction is persistent for at least 12 months. Reboot surgery seems to be favorable in preventing relapse compared to conventional mucosa sparing surgery in terms of relapse rates and in time to recurrence implicating the need for more complete surgery. No patients participating in these reboot studies suffered from any serious complications intra- or postoperatively, such as orbital damage, cerebral fluid leakage or major infections.

5 DISCUSSION

CRS is a common disease with significant impact on patients' quality of life, especially for patients with type 2 CRSwNP who often experience relapse of the disease after treatment. We have shown that the inflammation in CRSwNP is not limited to the polyps themselves, but is also present in the non-polypoid sinus mucosa. It is possible, that part of the reason for relapse after mucosal sparing surgery, is that this mucosa maintains an extensive inflammation which is not addressed during conventional surgery. Our results also show that all sinuses are equally inflamed, something that is supported by the findings in computed tomography studies after treatment with mAbs. These antibody-based drugs have previously been shown to reduce opacification in all sinuses (total Lund-Mackay scores) in severe CRSwNP patients[106-109] supporting the notion of ongoing inflammation in all sinuses.

In CRSwNP, there are geographical differences in the inflammatory background. A multicenter study has shown that European CRSwNP patients express predominantly type 2 inflammation whereas patients from Beijing show a mixed type 1/type 2/type 17 inflammatory pattern, patients from Japan show higher individual type 2 patterns compared to Beijing, but lower patterns than European patients[23]. A shift towards increased type 2 inflammation has been seen in Thailand[102], Korea[103] and China[104] over recent years. We can now show that a similar shift towards increased type 2 inflammatory parameters and an actual shift of endotype, described as an increase in IL-5 positivity in tissue, is seen in Western Europe. Interestingly, this shift is only seen in non-asthmatic, non-allergic patients; asthmatic patients already show a high expression of IL-5, which obviously is not subject to further increase. If the shift is caused by an altered microbiome, environmental factors or something else is still to be investigated. We know that patients with type 2 CRSwNP have a more severe form of CRSwNP and are more prone to relapse[11]. This observed increase in type 2 inflammation might mean that we will be facing more patients with severe and difficult to treat CRSwNP in the future, further illustrating the importance of being able to endotype these patients and to find novel treatment strategies.

Here we describe two different entities of CRS based on levels of IL-5 in tissues, IL-5 positive and IL-5 negative. IL-5 negative patients with and without nasal polyps are similar with no differences in levels of inflammatory markers or comorbidities, whereas IL-5 positive polyp and non-polyp patients express elevated levels of IgE and ECP compared to IL-5 negative patients. Within the IL-5 positive group, CRSwNP patients express elevated levels of IgE and ECP compared to CRSsNP patients and a higher proportion of CRSwNP patients have asthma and AERD comorbidity as well as presence of SE-IgE in polyp tissue. Interestingly, polyps appear in both groups, regardless of levels of type 2 markers,

as seen in non-type 2 CRSwNP[23, 24]. This suggests that the mechanism that drives polyp formation is independent from type or level of inflammation. It is possible that type 2 inflammation enhances the polyp disease once it has started, but the trigger might be something else.

Patients with asthma have elevated levels of type 2 markers in nasal polyp tissue and EBC. AERD comorbidity does not enhance this elevation, suggesting that asthma alone causes maximal inflammation. Nevertheless, a recent study has shown that patients with AERD had undergone more sinus surgeries than CRSwNP patients with asthma only (1.4vs 2.6)[110] suggesting that patients with AERD have a more severe form of CRSwNP that is not reflected in polyp tissue type 2 inflammatory markers.

In order to implement personalized treatment for CRSwNP patients, they have to be properly endotyped. Serum periostin is elevated in CRSwNP patients compared to CRSsNP patients and controls and serum periostin, serum IgE and serum SE-IgE can be used to identify the most severe cases of CRSwNP, i.e. patients with IL-5 and SE-IgE in nasal polyp tissue, with good sensitivity and moderate specificity. Except for serum IgE, these markers are not yet used in clinical practice. EBC is commonly used as a surrogate marker of tissue eosinophilia. In our data, there was a positive but poor correlation between EBC and inflammatory markers in tissue, when interpreting the results as suggested by Hinkle et al[100] with only IL-5 almost reaching up to a moderate positive correlation ($R=0.489$, $p<0.001$). Our prediction model reveals that elevated EBC (>300 cells/microL, the level used for treatment with mepolizumab in asthma) can, together with questions about asthma and AERD comorbidity, help to identify the least and the most severe cases of CRSwNP. After identification of type 2 inflammation in CRSwNP patients due to their comorbid asthma, an elevated EBC helps to identify tissue IL-5 positive CRSwNP patients without asthma/AERD/allergy, and can be very helpful in a clinical setting. However, EBC alone does not indicate the type or the severity of inflammation in the polyps. EBC is a difficult marker to use, since it can be affected by many other conditions, such as corticosteroid therapy, allergies, autoimmune diseases and parasite infections. A recent study showed an inverse relationship between EBC and oral GCS use[69], hence consideration must be taken when the EBC test is executed.

Recent studies have shown that monoclonal antibodies directed towards type 2 immune responses to be effective in decreasing polyp size and improving symptom scores in CRSwNP patients[81, 86, 92]. Dupilumab, affecting the IL-4/IL-13 pathway, reduces local inflammatory type 2 parameters in tissue and in nasal secretions. The reduction in nasal secretions was 78.8% for IgE and 38.6% for ECP. These results point to a local effect on eosinophilic activation and migration. Dupilumab will

possibly shorten the lifetime of eosinophils although dupilumab does not directly reduce IL-5. Treatment studies of mepolizumab (anti-IL-5) have not shown a consistent decrease in nasal ECP, total IgE or IL-5[92]. IL-4 has, unlike IL-5, the ability to promote the maturation of naïve T0 cells into Th2 cells and to upregulate IgE receptors on the cell surface of mast cells, basophils, B-lymphocytes and mononuclear phagocytic cells and thus to upregulate IgE-mediated immune responses[32]. This can possibly explain the differences between the two medications on local inflammatory parameters. Dupilumab (Dupixent®) is, the only mAb so far, approved for CRSwNP, in USA and Europe, but is not yet in use in Sweden. Treatment with mAbs are expensive (yearly cost for one patient is between 120 000 – 300 000 SEK) and possibly a life-long treatment is required to prevent relapse of the disease, and yet, one does not know which patients may respond.

Surgical approaches in CRSwNP have differed, ranging from very extensive[73] to minimal, with only opening the natural ostium and preserving the mucosa in order not to disturb the mucocilliary clearing[74, 75]. Despite different techniques, relapse in severe type 2 CRSwNP after surgery is common. Reboot surgery addresses, unlike the conventional mucosa sparing surgery, the non-polypoid inflamed tissue in all sinuses, hence removing inflammatory cells and other triggers, such as *Staph. Aureus* that, when left, can cause the inflammation to persist. This is probably part of the success in terms of relapse rates, where reboot is superior to mucosa sparing surgery. Despite extensive surgery, reboot patients report significantly improved SNOT-22 scores one year after surgery compared to patients undergoing mucosa sparing surgery. Reboot surgery positively affects local inflammation in the nose, measured as a decrease in levels of type 2 inflammation. This decrease is in the same range as reported after treatment with dupilumab[111]. The decrease in inflammatory parameters persists for at least 1 year after surgery, but does not decrease to the levels of healthy individuals. Even so, patients do not relapse in their disease at the same speed[112] as after mucosa sparing surgery. A previous study has shown that inferior turbinates from severe CRSwNP patients contain elevated levels of type 2 inflammatory markers compared to healthy inferior turbinates[113]. Here, we show that middle turbinates from CRSwNP also express elevated levels of IgE and ECP, although the mucosa is not prone to form polyps. This inflammation in the middle and inferior turbinates can explain why inflammatory levels in nasal secretions don't decrease to the same levels as in healthy controls after surgery.

The reboot technique is controversial. Removing all mucosa down to the periosteum is thought to cause severe complications and scar formation post-operatively. In our patients we saw healthy mucosa covering the sinus walls within 4-6 weeks post-operatively, and this mucosa showed all elements of normal structured ciliated epithelium with activated goblet cells[112]. It is well known that resection of large areas of sinus mucosa often is needed in cases of malignant sinus tumors or

inverted papilloma, where appropriate wound healing has been observed[114, 115]. The positive post-operative healing effect in CRSwNP may come from the fact that type 2 immune reactions disturb reepithelization[14], and the complete removal of the sinus mucosa therefore can be regarded as an advantage for wound healing. It is also likely that the healing process in our patients is influenced by the treatment with doxycycline postoperatively. Doxycycline, a tetracycline antibiotic, is known to affect different critical aspects of the wound healing process[116, 117]. Doxycycline is a potent inhibitor of synthesis and effects of matrix metalloproteinases (MMP)[118]. Elevated levels of MMP-9 in the preoperative and postoperative period have been shown to be associated with poor wound healing in CRS[119]. Huvenne et al. has shown improved wound healing in CRS patients undergoing frontal sinus surgery when treated with doxycycline releasing stents compared to placebo stents[120]. Doxycycline coated tracheal stents have been shown to reduce tracheal inflammation and fibrosis in a rabbit model[121] and doxycycline inhibits staphylococcal Exotoxin induced T-cell proliferation and cytokine release[122]. All patients in our study, regardless of treatment with reboot or non-reboot surgery, were treated in the same way, hence doxycycline does not likely influence the recurrence rates when comparing both groups. In patients with severe CRSwNP, new treatment strategies should be considered, including treatment with mAbs and reboot surgery.

6 CONCLUSIONS

- The inflammation in CRSwNP appears to be widespread involving not only polyp tissue by also the seemingly heathy tissue that lines all sinus cavities. These results also indicate that the formation of polyps, at least in part, is driven by mechanisms not directly related to the type and extent of tissue inflammation.

- A shift towards type 2 inflammation is evident in non-asthmatic, non-allergic patients with CRS during the past 8-10 years. This swing is more pronounced in CRSwNP than in CRSsNP. This indicates that severe CRS cases might be more common in the future.

- Serum periostin, IgE and SE-IgE can be used as biomarkers to identify type 2 CRSwNP with reasonable sensitivity and specificity. The levels of EBC correlates poorly with type 2 inflammatory markers in nasal polyp tissue. However, used together with questions about asthma, allergy and AERD they can help identifying the most severe cases in a clinical setting.

- Dupilumab reduces levels of type 2 inflammation in nasal polyp tissue and in nasal secretions demonstrating a local effect on eosinophilic activation, survival and migration.

- Reboot surgery appears to be superior to a more traditional surgical approach when it comes to symptom reduction and increase in quality of life in severe cases of CRSwNP. Reboot surgery reduces the type 2 inflammatory response, 12 months after surgery, in a magnitude well corresponding the effects induced by dupilumab.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kronisk rhinosinuit (CRS) är en inflammation i näsa och bihålor med symtom som varar minst 12 veckor. CRS kännetecknas av nästäppa, rinnsnuva, nedsatt lukt och smak samt ansiktssmärta. Ungefär var tionde europé drabbas någon gång av denna sjukdom, något som orsakar stora kostnader för både individer och samhälle. Patienter med CRS besväras även, utöver symtomen i näsan, av andra symtom, som sömn- och koncentrationssvårigheter. De upplever också en sänkning av sin livskvalitet i paritet med t.ex. hjärtsviktspatienter.

CRS delas upp i sjukdom med och utan näspolyper (CRSwNP respektive CRSsNP). Vid båda tillstånden kan den underliggande inflammationen i sin tur delas in i typ 1 och typ 2. Typ 2-inflammation domineras av eosinofila celler, ses företrädesvis i västvärlden och är ofta associerad med astma, medan typ 1 domineras av neutrofila celler och är starkt representerad i Asien. Båda typerna kan genom laboratorieundersökningar av nässlemhinna och polyper delas upp i ett flertal undergrupper, s.k. endotyper. Vilken endotyp patienten tillhör säger något om hur patienten kommer att svara på behandling.

Traditionellt behandlas CRS med kortisonnässpray och näsköljningar samt vid behov kirurgi. Vid svår sjukdom kan de kirurgiska ingreppen bli många. Svår astma har sedan några år behandlats med en ny form av läkemedel, monoklonala antikroppar, även kallade biologiska läkemedel. Dessa läkemedel riktar sig direkt mot enskilda cellers inflammatoriska svar och på så sätt sjukdomens endotyp. Dessa monoklonala antikroppar fungerar speciellt bra vid typ 2 astma. De har därför, med framgång, nyligen även testas vid CRSwNP av typ 2.

Målet med föreliggande avhandling är att kartlägga inflammationsbiologin bakom CRS endotyper, och med detta som utgångspunkt finna lämpliga laboratorieprov (biomarkörer) som i kombination med samsjuklighet, som astma och allergi, kan ge prognostisk och terapeutisk vägledning. Vidare utvärderas ett nytt kirurgiskt angreppssätt (reboot) samt en ny biologisk behandling vid CRSwNP.

Delarbete I och II undersöker om biomarkörer i kombination med samsjuklighet kan användas för att förutsäga speciellt svåra former av CRSwNP. Avhandlingen lyfter här fram tre blodprov som var och ett för sig, eller i kombination, skulle kunna användas för detta ändamål: Periostin, Immunoglobulin E (IgE) och specifika IgE antikroppar mot staphylococcus aureus (SE-IgE). Vi visar också att den traditionella markören eosinofiler i blod korrelerar dåligt med typ 2 markörer i näspolypvävnad, men att eosinofiler i blod tillsammans med frågor om astma och allergi kan hjälpa till att identifiera de svårast sjuka CRS patienterna. I delarbete

II visas även att en allt större del av CRS patienterna i Europa övergått från typ 1 till typ 2, något som tidigare har varit känt i Asien.

I delarbete III-V undersöks nya behandlingsformer. I delarbete III visas hur en ny monoklonal antikropp, Dupilumab, sänker nivåerna av typ 2-markörer i vävnad och sekret. Detta tyder på en lokal effekt på de eosinofiler i vävnaden som driver sjukdomen. Delarbete IV är en retrospektiv studie, som jämför en ny, mer extensiv, kirurgisk metod (reboot) med konventionell kirurgi vid CRSwNP. Vid reboot tas all slemhinna i bihålorna bort, inte bara polyper som vid konventionell bihålekirurgi. Patienter som genomgått reboot kirurgi hade lägre recidivrisk vid tvåårsuppföljning jämfört med patienter som opererats på traditionellt sätt. De beskrev också en mer uttalad symtomförbättring än de traditionellt opererade patienterna. I delarbete V visade vi att inflammationen vid CRS inte är begränsad till själva polyperna utan omfattar all slemhinna som utkläder bihålorna. Detta förklarar sannolikt varför reboot-kirurgi, där all slemhinna avlägsnas, minskar risken för återfall. Avslutningsvis presenteras data som visar att reboot-kirurgi reducerar inflammationsbilden i bihålan på samma sätt som behandling med Dupilumab samt att denna sänkning kvarstår 12 månader efter operationen.

Sammanfattningsvis kastar denna avhandling nytt ljus över patofysiologin vid CRSwNP, där ett skifte mot svårare sjukdom ses i Europa. Avhandlingen visar att biomarkörer och klinisk karakteristik kan hjälpa till att identifiera de svårast sjuka. Den ger även en ökad förståelse för mekanismen bakom behandling med monoklonala antikroppar samt introducerar en ny kirurgisk metod och förklarar bakgrunden till varför mer extensiv kirurgi är framgångsrik vid svårbehandlad CRSwNP.

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